FINE STRUCTURE OF *GROMIA OVIFORMIS* (RHIZOPODEA : PROTOZOA)

BY

RONALD HENDERSON HEDLEY & JAMES ST. JOHN WAKEFIELD

Department of Zoology, British Museum (Natural History)
Cromwell Road, London, S.W.7

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By R. H. HEDLEY & J. St. J. WAKEFIELD

CONTENTS

SYNOPSIS

Four shell-components are recognized—the outer wall, honeycombed membranes, fibrillar material and oral capsule. The outer wall is proteinaceous and finely fibrillar, with an outer electron-dense layer characterized by a high polysaccharide content. The whole structure is perforated at intervals by wall canals whose possible functions are discussed. The honeycombed membranes have an hexagonal architecture, and in cross section appear striated; the relationship of the striations to the hexagonal structure is illustrated. The oral capsule is composed mainly of polysaccharide microtubules, and its junction with the other shell-components is described. The significance of the amino acid hydroxyproline being present in the shell is discussed. A conventional plasma-membrane occurs below the fibrillar material of the shell.

The cytoplasmic organelles discussed in detail include the microbodies, Golgi apparatus, secretion granules and nuclei. Microbodies have a variable morphology and a simple tubular nucleoid. The Golgi apparatus is considered to be concerned with the manufacture of wall-material. The pseudopodia are characterized by the absence of organelles or any other recognizable structures; they are limited by a plasma-membrane and contain ground plasm.

Gametes possess a single mucronate flagellum, a single nucleus, two basal bodies, mitochondria, fat droplets and two enigmatic organelles.

INTRODUCTION

The widely-distributed, shallow-water, marine rhizopod *Gromia oviformis* Dujardin, 1835, has attracted the attention of protozoologists because of its relatively large size—up to 5 mm.—uncertain systematic position (Arnold, 1952; Hedley, 1958) and suggested similarity to some of the Chitinozoa, a palaeozoic group of fossils (Collinson & Schwalb, 1955; Hedley, 1962).

The present paper is a detailed account of the fine structure of the adult organism and the gamete and it clarifies certain problems of shell-structure raised by Hedley (1960) and Hedley & Bertaud (1962).

MATERIAL AND METHODS

Live animals were collected from the intertidal zone at Wembury Bay, Plymouth, England, where they live in abundance attached to protected rock-faces or the holdfasts of *Laminaria*. After collection they were placed in Foyn's Erdschreiber medium (Arnold, 1954) in plastic containers, 2 inches in diameter, and kept at room temperature, 18–20° C. Under these conditions animals live for up to 6 months, but do not appear to feed or grow despite being offered various flagellates and diatoms. Some animals, shortly after collection, liberated gametes.

Electron microscopy.—Gametes for shadowed whole-mounts were prepared by the method described by Manton (1964), in which a drop of sea-water containing gametes is placed on a carbon-coated grid. A petri-dish containing a few drops of 2% osmium tetroxide is inverted over the grid for 1½–2 minutes. The vapour fixes the gametes, which fall onto the carbon film; the excess liquid is removed with filter paper and the grid is allowed to dry. The salt deposit is removed by several washes of distilled water and the grid is dried again. These preparations are shadowed with gold/palladium at an angle of approximately 30°. For the examination of gamete ultrastructure the creamy peripheral layer of cytoplasm, characteristic of an animal about to liberate gametes, is pipetted off and squirted into fixative. After fixation the gametes are centrifuged to form a pellet, and in subsequent procedures they are handled as a single piece of tissue. The standard fixing and embedding procedure used for both gametes and adult animals is as follows. Specimens are fixed in 4% glutaraldehyde in 0·1 M cacodylic acid buffer with 0·25 M sucrose, and post-fixed in Caulfield's osmium tetroxide. After rapid ethanol dehydration, specimens were embedded in Epon 812. For preservation of pseudopodia the following fixatives were used: Palade's osmium tetroxide; Caulfield's osmium tetroxide; 2% osmium tetroxide in sea-water; 2% osmium tetroxide in 0·2 M cacodylic acid buffer with 0·4 M sucrose; 4% glutaraldehyde in sea-water followed by 2% osmium tetroxide in sea-water; 4% glutaraldehyde in 0·15 M cacodylic acid buffer with 0·3 M sucrose followed by 1% osmium tetroxide in 0·15 M cacodylic acid buffer with 0·4 M sucrose and 0·3 M glucose; 5% acrolein in 0·2 M phosphate buffer with 0·3 M sucrose followed by 1% osmium tetroxide in 0·2 M phosphate buffer with 0·3 M

sucrose and 0·2 M glucose; and the picric acid-formaldehyde mixture of Stefanini *et al.* (1967). Sections were cut with a diamond knife on a Porter–Blum ultramicrotome, stained with a saturated alcoholic solution of uranyl acetate followed by Reynold's lead citrate. For the demonstration of polysaccharide the methods of Rambourg (1967) and Thiery (1967) were used. Sections were examined on an A.E.I. EM 6B microscope operating at 60 kV, and recorded on Ilford N50 plates.

Amino acid analysis.—Approximately 3,000 specimens were used. After being picked off rock-surfaces, whole animals were placed in distilled water so that the resultant osmotic effect caused most of the cytoplasm to be squeezed out of the shell. Each empty shell was cut into two or three pieces and placed for two hours in an ultrasonic cleaner. Several shells were embedded for electron microscopy, and on examination the outer wall and honeycombed membranes were still intact, while all contaminating material had been removed. The clean shells were ground with powdered glass in either 6N/HCl or saturated $Ba(OH)_2$. Extracts from both were prepared by standard hydrolysis procedures and subjected to two dimensional chromatography on thin-layer silica gel plates, using the following solvent systems: (a) *n*-butanol: acetic acid: water (60 : 20 : 20)/phenol; (b) chloroform : methanol : 17% ammonia (2 : 2 : 1)/phenol. The chromogenic agent used was ninhydrin. After reference to standard maps comparable mixtures of amino acids were prepared and run in parallel with the extracts.

SHELL

Previous work.—The shell of *Gromia* was first described by Bütschli (1894), who noted that the single opening could be closed by the collar. A more detailed description by Awerinzew (1903) indicated that the wall was a two-layered structure, with an outer perforate and an inner structureless layer. Jepps (1926) confirmed this description, adding that the outer perforate part of the wall was composed of a mosaic of irregular prismatic rods, and was resistant to hydrolysis. She concluded from a number of tests that the wall was composed of pseudochitin, a term introduced by Awerinzew (1907) when describing the composition of fresh-water testacean shells. Apart from a text-figure by Jepps (1926), showing the junction between wall and oral capsule, the relatively complex oral region had not been studied until Arnold (1952) published a diagram of a dissected oral region. The shell was next described as a single-layered radially perforate tectinous structure, with the inner structureless layer of previous authors being interpreted as a layer of non-granular cytoplasm (Hedley, 1960). The same author described the oral region in detail and gave an account of the opening and closing movements of the oral capsule. The shell was considered to be composed of mucoprotein with an unexpected high content of organically bound ferric iron, whilst the oral capsule was shown to be predominantly acid mucopolysaccharide. Hedley & Bertaud (1962), in a study of shell ultrastructure, confirmed the perforate nature of the outer wall, and described the fine structure of the wall as having a sponge-like texture. They also reported a unique system of honeycombed membranes, but did not locate a conventional plasma membrane between the cytoplasm and the shell. The oral capsule was shown to be made up

of a mass of microtubules arranged in a well-organized but complex pattern. Some of the problems which arose from the work of Hedley & Bertaud (1962) are considered in the present account. These include the relationship between the oral capsule and the shell-wall, the function of the canals which perforate the wall, the position of the plasma membrane, and the position of the bound ferric iron.

The component parts of the shell, which are recognized and discussed in the present paper, are, beginning from the exterior, the outer wall invested by an electron-dense layer, the honeycombed membranes, the fibrillar material, and in the oral region the oral capsule (Text-fig. 1, Pl. 2, fig. A, Pl. 3, fig. A).

Outer wall.—The wall is composed of a mass of fine fibres 5–6 nm. thick (Pl. 3, fig. B), and is perforated at intervals by wall canals (Pl. 2, figs. B and C). In cross sections of the wall the fibres are aligned parallel to the shell-surface (Pl. 3, fig. B). In tangential section the fibres are arranged at random, except in the vicinity of wall canals where they are arranged concentrically around the canal to a depth of about 0·1 μm (Pl. 2, fig. B).

The electron-dense points, 4 nm. in diameter, visible in cross sections are probably fibres running at right angles to the plane of section, rather than condensations of some other electron-dense material. The concentration of these points along the edge of the canals in longitudinal section (Pl. 2, fig. C), where many of the fibres are at right angles to the plane of section (Pl. 2, fig. B), is consistent with this view.

The outer surface of the wall is limited by a strongly electron-dense layer, 10–16 nm. thick (Pl. 2, figs. A and C, Pl. 3, figs. A and B). In an attempt to determine the origin of this electron density some animals were fixed in glutaraldehyde, not post-fixed in osmium tetroxide, and not stained with uranyl acetate or lead citrate after sectioning. In these, the outer layer was still electron dense, but less so than in normally-prepared sections, indicating that the electron density is not entirely due to introduced heavy metals, and consequently not entirely due to the presence of unsaturated fatty acid (Korn, 1966). Earlier studies have shown that the outer wall of *Gromia* has a high content of bound ferric iron (Hedley, 1960). The modification of Perl's method for ferric iron in electron microscopy (Thiéry, 1962) was used to see if the iron was concentrated in the outer electron-dense layer. Unfortunately, in this laboratory, the method does not produce satisfactory results, as an even precipitate is deposited on the section over both the specimen and embedding medium. However, the electron density present in specimens which had not come into contact with heavy metals during preparation indicates that the electron-dense layer may have a high content of ferric iron. Using the silver methenamine stain of Rambourg (1967), and Thiéry (1967), the electron-dense outer layer reacts positively for polysaccharide (Pl. 6, fig. B).

Canals which perforate the outer wall are present throughout the shell and are not restricted to any particular area. In cross section they are usually circular, 0·2–0·3 μm in diameter (Pl. 2, fig. B) and vary from straight cylinders to irregular passages with extensive blind cisternae. The canals open freely on the inner surface of the outer wall (Pl. 2, fig. C), but the opening on the outer surface is reduced to a small pore (Pl. 3, fig. B and D). The pore, approximately 85 nm. in depth and 35 nm. in diameter, is formed by an open-ended invagination of the outer electron-dense

layer. The function of the wall-canals is still unresolved, although the suggestion by Hedley & Bertaud (1962)—that they are pores through which cytoplasm can pass—now appears to be untenable. The cytoplasm lies beneath the honeycombed membranes, and to have access to the outside through the wall-canals, it would be

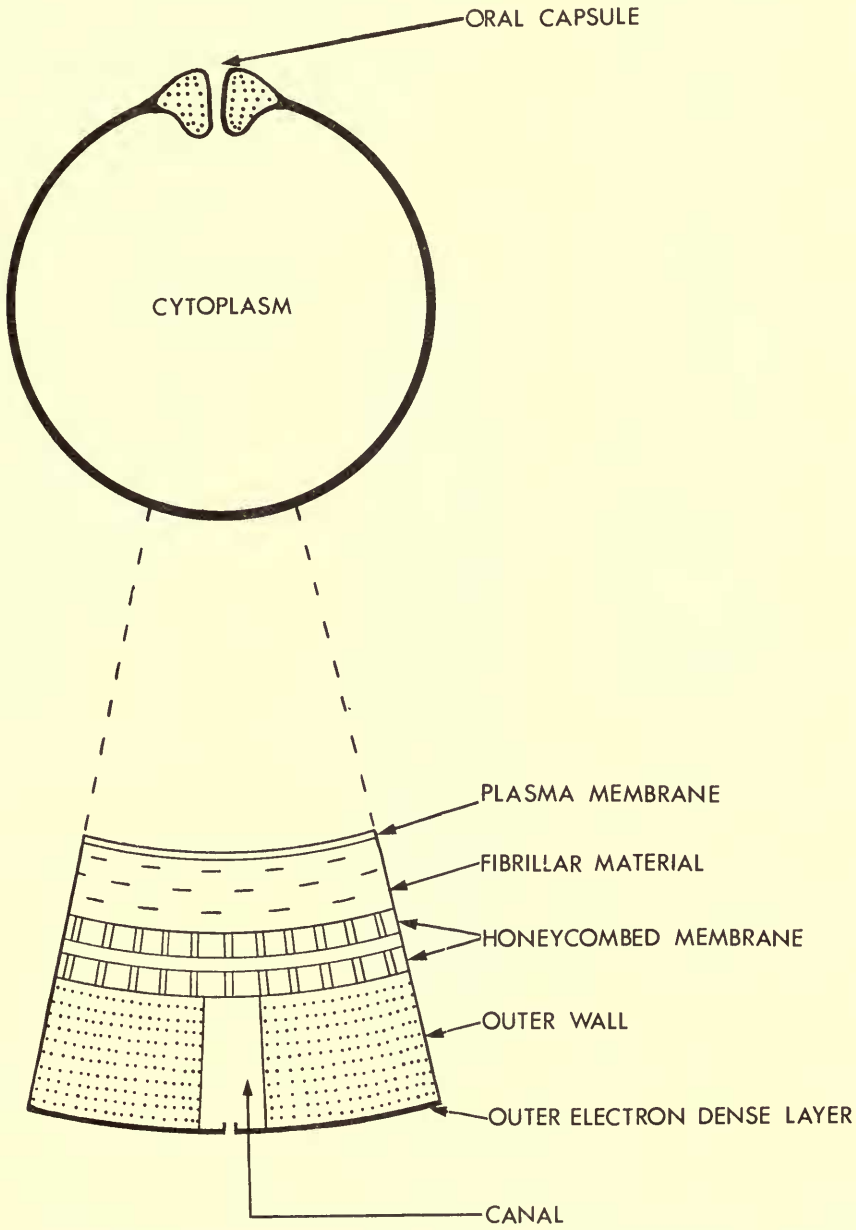

FIG. 1. Diagram of the shell of *Gromia oviformis* showing the position of the oral capsule and details of wall-structure discussed in the text.

necessary to have gaps in the honeycombed membranes. These have not been seen, and cytoplasmic material has not been detected in the wall-canals. Another possible function of the wall-canals is to allow the release of gametes to the exterior. Adults are often half buried in mud and debris, which is loosely cemented together, with the oral region attached firmly to the substrate. During gametogenesis the cytoplasm separates from the stercomata and xanthosomes which occupy the lower half of the shell, the maturing gametes developing in the upper half (Hedley, 1962). In this position there is no escape route for the gametes through the mouth and the wall-canals could provide a possible exit. The head of the gametes, however, measure 2–3 μm while the wall canals are only 0·2–0·3 μm, moreover the honeycombed membranes separate the gametes from the outer wall. The passage of gametes through the honeycombed membranes and wall-canals could be facilitated by enzyme action partially breaking down the honeycombed membranes and outer wall at gametogenesis. This suggestion is supported by the observation that shells of animals which have undergone gametogenesis are much weaker than normal and less strong than those shells of heat-killed animals, which have been left in non-sterile sea-water for many months (Hedley, 1962).

Honeycombed membranes.—Moving inwards from the outer wall the next component of the shell is the complex of honeycombed membranes (Text-fig. 1, Pl. 2, fig. A, Pl. 3, fig. A). These lie parallel to the outer wall in sheets, measuring 20–50 nm. thick and 7–15 μm across, forming an interleaved stack of up to 15 membranes. The structure of a membrane is best seen in tangential section, which gives a plan view (Pl. 4, fig. A). The membrane is composed of an hexagonal array of cylinders 10–20 nm. in diameter, with a wall-thickness of 2–2·5 nm., spaced 10 nm. apart. Each cylinder is connected to the six surrounding cylinders by a septum 2–2·5 nm. thick, in the middle of which is an electron-dense spot 3–4 nm. Occasionally a tangential section may contain more than one membrane. When this occurs the simple hexagonal pattern of each interacts producing Moiré patterns (Pl. 3, fig. C). Similarly produced Moiré patterns have been described from negatively stained bacterial membranes (Glauert, 1966).

When cut in cross section (Pl. 2, fig. A, Pl. 3, fig. A, Pl. 4, fig. B) the membranes appear as banded ribbons 20–50 nm. thick. Four types of banding may be distinguished: Type 1. An alternating sequence of 10–12 nm. thick light bands and 7–8 nm. thick dark bands, with an interperiod length of 20 nm. (Pl. 3, fig. A); Type 2. An interperiod length of 20 nm. composed of the following sequence; dark 3·5 nm., light 6·5 nm., dark 3·5 nm., light 6·5 nm. (Pl. 3, fig. A). That the repeated period is 20 nm. rather than 10 nm. composed of dark 3·5 nm., light 6·5 nm. bands is difficult to detect and is due to every second light band being slightly less electron dense than every first light band; Type 3. An interperiod length of 6 nm. composed of alternating light and dark bands 3 nm. thick (Pl. 4, fig. B); Type 4. An homogeneous non-banded ribbon (Pl. 3, fig. A). The banding pattern is constant throughout the length of any one membrane. The frequency of occurrence of banding patterns estimated from an examination of all available micrographs is approximately, type 1, — 50%, types 2 or 3, — 45%, and type 4, — 5%.

The banding patterns produced in membrane cross sections are due to the arrange-

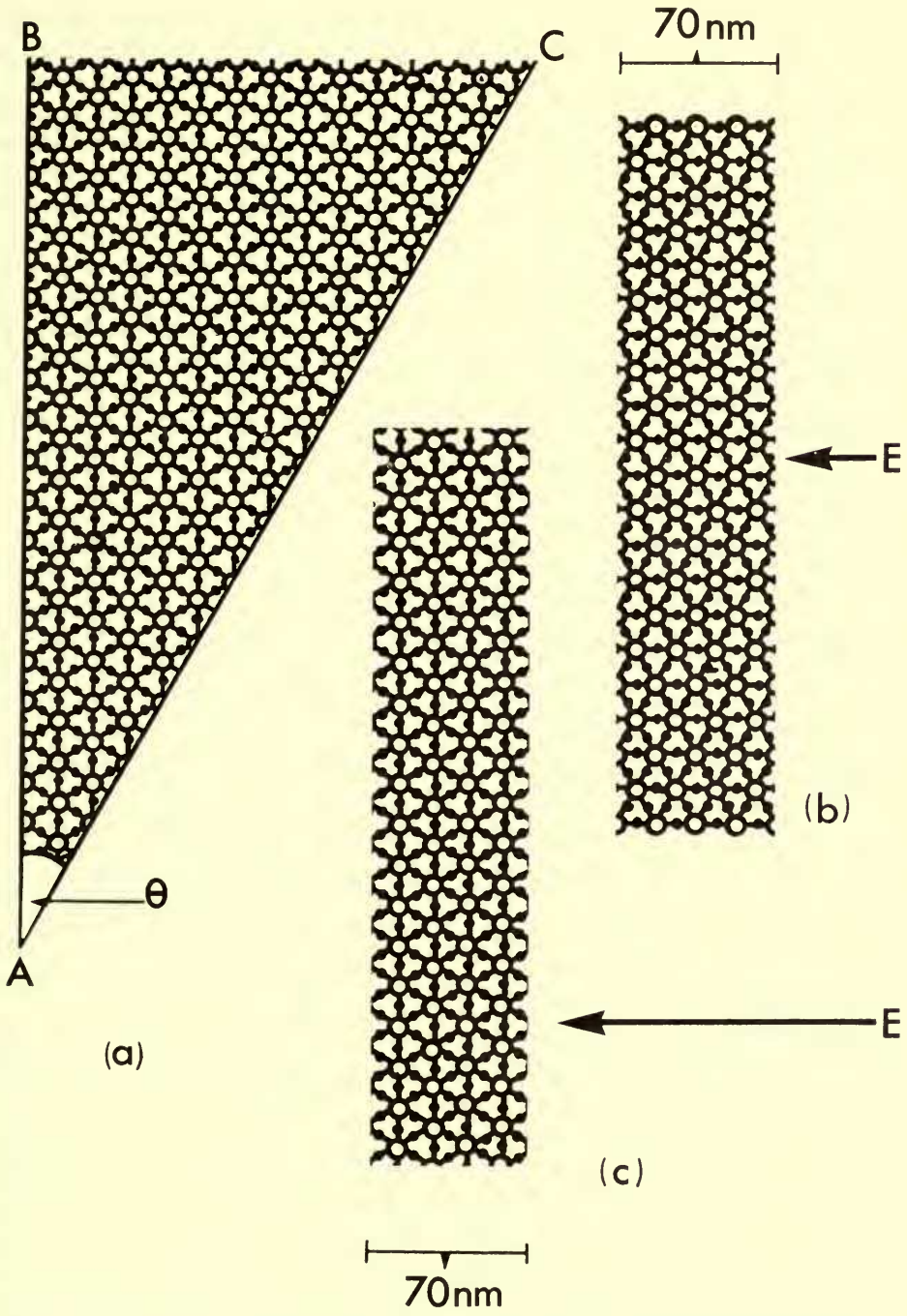

FIG. 2. Plan view of a honeycombed membrane: for discussion see text. E, indicates the direction of the electron beam.

ment of the cylinders in that section, and can be related to the plan view of the membrane in the following way. In plan view the cylinders are arranged in a strict hexagonal array (Pl. 4, fig. A). There are three axes of symmetry with a 60° angle between each. Each 60° segment is identical and bordered by two axes, and can be divided into two symmetrical halves 30° each (Text-fig. 2a). When a membrane is cut in section the direction of section is at a constant angle to the axes throughout the extent of that membrane. If an average section thickness of 70 nm. is assumed (Williams & Meek, 1966), then Text-fig. 2b represents a plan view of such a section in the direction of AC (Text-fig. 2a) i.e. the bisector of a 60° segment. When this is viewed in cross section, in the electron microscope, the membrane appears as an alternating sequence of dark and light bands, i.e. type 1 banding pattern (Pl. 3, fig. A). That this banding is due to the arrangement of the cylinders, is clearly seen in Pl. 4, fig. C. When the angle θ decreases (Text-fig. 3a and c—a section in the direction AB where $\theta = 0°$) the banding pattern will change and one of the types 2, 3 or 4 will be produced. The particular arrangement of cylinders which produces any of these banding patterns has not been deduced.

Fibrillar material.—The outer wall, honeycombed membranes and plasma membrane are sometimes in close contact, but more usually they are separated from each other by a fine fibrillar substance (Pl. 2, fig. A, Pl. 3, fig. A). This is morphologically similar to that material comprising the outer wall and occurring in the wall-canals. Further, there is a more dense amorphous substance, which may be either condensations of the fibrillar material or an entirely different substance. The function of this material is unknown, although part of the fibrillar material may be a precursor of the outer wall. There is often a region on the inner face of the outer wall which is slightly more electron dense than the rest of the wall (Pl. 2, fig. C), and this is presumably the zone of accretion. It is unlikely that the wall grows by addition of material to the outer surface, because in *Gromia* there is no extramural layer of cytoplasm bathing the shell, as there is in allogromoid foraminifera (Hedley, 1964).

Amino acid composition.—The following amino acids were detected; alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, leucine/iso-leucine, lysine, ornithine, phenylalanine, proline and valine. The somewhat surprising presence of hydroxyproline was checked and confirmed. Hydroxyproline is usually considered to be an amino acid unique to the skeletal protein collagen. There are, however, some exceptions to this generalization. One is in certain plant cell-walls which are non-collagenous and where hydroxyproline comprises 13% of the amino acids present (Lamport & Northcote, 1960). A second probable exception occurs in the nematocyts of both *Hydra* and *Physalia*, where a hydroxyproline content of 22–31% is reported (Lenhoff *et al.*, 1957, Lenhoff & Kline, 1958), and where in *Hydra* collagen has not been demonstrated by electron microscopy (Slautterbach, 1963). As there is no collagen demonstrable in the fine structure of the shell of *Gromia*, the hydroxyproline almost certainly originates from a non-collagenous protein. Another possibility, although somewhat unlikely, is that the honeycombed membranes are composed of a collagen with an atypical fine structure. A collagen organized in an hexagonal pattern has been described by Jakus (1956). This is Descemet's membrane from the inner surface of the cornea of a variety of vertebrates. The

filaments making up this membrane do not possess the typical banded pattern of collagen, but their collagenous nature has been established by chemical analysis and x-ray diffraction. There are considerable differences between this membrane and those found in *Gromia*: the spacing of the hexagonal lattice in Descemet's membrane is 100 nm., whilst in the honeycombed membranes in *Gromia* it is 20 nm.; the nodes where the axes join in Descemet's membrane are solid, in the honeycombed membranes they are hollow cylinders; in Descemet's membrane the individual lamellae are less than 5 nm. thick, in the honeycombed membranes they are 30 nm. thick. If the honeycombed membranes are collagenous, it is difficult to see how the tropocollagen molecule (300 by 5 nm.) makes this hexagonal pattern where the longest straight length is only 10 nm.

Oral capsule.—The extracellular oral capsule is composed of outer wall material enclosing a mass of microtubules (Text-fig. 2, Pl. 5). There is no definite junction between wall and oral capsule, the most obvious change in the region being the disappearance of the honeycombed membranes (Pl. 5). The outer wall thickens and bifurcates, the main part going along the outer face of the oral capsule, the much smaller branch lining the inner face. Between the two branches, where they divide, there is a thickening of outer wall materal. The extension of the wall along the outer surface of the oral capsule covers approximately two-thirds of the capsule. Near its edge the outer wall tapers to a point and the outer surface is crenated. The thinner extension of the wall along the inner surface of the oral capsule also continues for about two-thirds of the length of the capsule. It is these two extensions of the outer wall which stain strongly with certain basic dyes (Hedley, 1960, fig. 2F).

The strongly electron dense layer (Text-fig. 3, Pl. 5), is prominent in the oral capsule; it lines both surfaces of the outer extension of the outer wall, and it lines most of the microtubular mass. In the region of the microtubular mass, between the end of the electron-dense layer and the end of the outer wall extension, there is a 1 μm thick layer composed of a poorly-defined fibrillar material (Pl. 4, fig. D). The fibrillar material of the shell lines the base of the oral capsule at the end of which it merges with a similar layer lining the pseudopodia.

The main part of the oral capsule is composed of microtubules 25–30 nm. in diameter and of filaments 5–6 nm. thick which lie between the tubules (Pl. 4, fig. D) When the capsule is cut in vertical section (Text-fig. 3, Pl. 4, fig. D) the microtubules are always cut in cross section. When the capsule is cut tangentially the tubules are cut in tengential section and are arranged in a chevron pattern (Pl. 6, fig. B). A three dimensional reconstruction of the arrangement of the microtubules from this evidence has not been possible, but the predominant direction of the tubules is circumoral. This microtubular part of the oral capsule is mainly acid mucopolysaccharide with little or no lipid or protein (Hedley, 1960). Using the silver methenamine technique (Rambourg, 1967) both the microtubules and fibrils stain strongly, indicating a polysaccharide composition (Pl. 6, fig. B). Although the microtubules are morphologically similar to cytoplasmic microtubules described from animal and plant cells (Porter, 1966) they are basically quite different. The microtubules of the oral capsule are extracellular, cytoplasmic microtubules are intracellular. Cytoplasmic microtubules are proteinaceous (Shelansky & Taylor, 1967), those of the

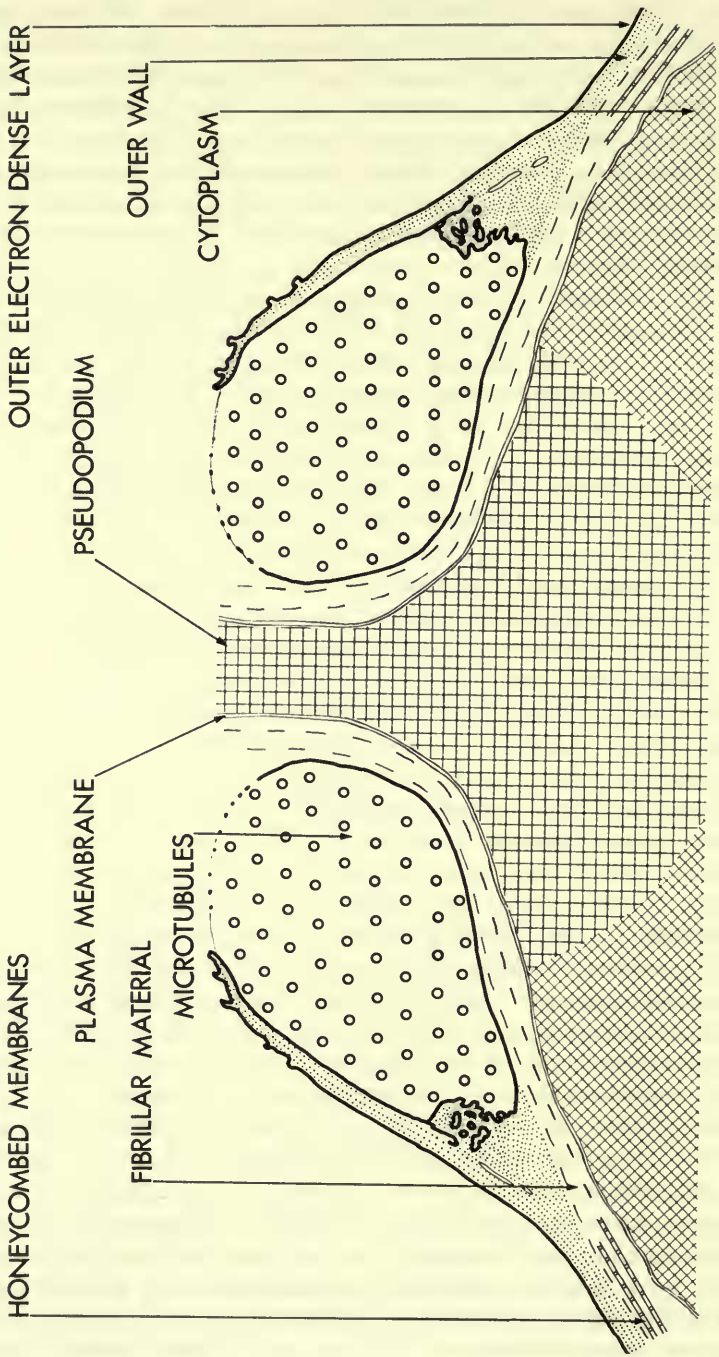

Fig. 3. Diagram of the oral capsule and opening through which pseudopodia are extruded.

oral capsule are composed of polysaccharide. The substructure of the microtubules in the oral capsule is unknown, and it is not possible to compare them, at this level, with cytoplasmic microtubules whose substructure has been resolved (Behnke & Zealander, 1967).

CYTOPLASM

Previous work.—Several protozoologists have described some aspects of the cytoplasmic structure of *Gromia* (Reichert, 1865, Schultz, 1866, Rhumbler, 1894, Bütschli, 1894, Schaudinn, 1894, Zarnik, 1907, Awerinzew, 1910), but it was Jepps (1926) who made the first general study. The next account of importance was concerned primarily with fine structure (Hedley & Bertaud, 1962). This, like other studies which had preceded it, was hampered by difficulties inherent in sectioning the animal, due to the presence of brittle xanthosomes and ingested sand-grains. Inadequate fixation has also limited the results obtained from previous investigations. Consequently certain features of cytoplasmic organization have remained unresolved, and further investigation of these, in particular the structure and position of the plasma membrane, the fine structure of the pseudopodia and general cytoplasmic ultrastructure, has been the chief aim of the present study.

Plasma-membrane.—A conventional plasma-membrane (Robertson, 1963) was not detected by Hedley & Bertaud (1962), and they suggested that the honeycombed membranes might be an unusual form of plasma-membrane. Later, this possibility was extended when it was suggested that the honeycombed membranes might represent a high permeability state of a normal cell-membrane in response to chemical or physical stimulus (Bertaud & Hedley, 1963). This view was expressed in the light of normal and conventional cell-membranes of red cell ghosts proving to have an hexagonal architecture after saponin treatment (Dourmashkin *et al.*, 1962). Kavanau (1963, 1965) has also suggested that the honeycombed membranes in *Gromia* may be a plasma-membrane, interpreting them, with reference to his membrane model system, as membranes fixed in the open configuration (Kavanau, 1963). These postulations are now shown to be incorrect as a conventional plasma-membrane is now demonstrated, lying below the honeycombed membranes (Pl. 2, fig. A, Pl. 3, fig. A, Pl. 8, fig. C). The membrane is tripartite, 8 nm. thick, the component lamellae measuring 2·5 nm. (electron dense), 3 nm. (electron lucid), 2·5 nm. (electron dense). It delimits the protoplasm, including the pseudopodia.

Kavanau (1965) also suggested that the honeycombed membranes may have been constructed upon a template of a plasma-membrane in the open configuration. As we have found no indication of the site of honeycombed membrane formation we can make no comment on this suggestion.

Nucleus.—Nuclei are numerous, about 6 μm in diameter, and distributed throughout the cytoplasm. The nuclear matrix is finely granular (Pl. 6, fig. A) and condensations of chromatin or heterochromatin have not been seen. There is a single nucleolus of the heterogenous type, usually with a central region of electron-dense granules, 20 nm. in diameter, and a peripheral ring of more tightly-packed smaller granules (Pl. 7, fig. A). A conventional nuclear membrane is present composed of

two tripartite membranes, each 6 nm. in width. Nuclear pores are numerous and made conspicuous by the presence of an electron-dense material which fills the pore (Pl. 6, fig. A). When cut in tangential section the pores of the nuclear membrane appear to have an electron-dense core 20 nm. in diameter, surrounded by a less dense ring 50 nm. in diameter, and then a dense margin 90 nm. in diameter (Pl. 7, fig. B). The closest centre to centre spacing observed is 100 nm., and there are approximately 13–15 pores per μm^2. These figures are consistent with those given by Yoo & Bailey (1967) for nuclei from a variety of cells. Ribosomes are present on the cytoplasmic side of the outer nuclear membrane and in tangential section they are seen grouped as polysomes (Pl. 7, fig. B). Continuity between the outer nuclear membrane and the endoplasmic reticulum is common.

Endoplasmic reticulum and ribosomes.—Endoplasmic reticulum is not extensive in *Gromia* and usually occurs in regions free from cytoplasmic particles and organelles. The cisternae are distended and contain a material identical in appearance to the surrounding ground plasm (Pl. 9, fig. C). The cytoplasmic side of the endoplasmic reticulum membrane is studded with ribosomes, these are often grouped as polysomes which occur in crescents 8–12 ribosomes in length (Pl. 10, fig. B). Polysomes free in the ground plasm have not been found.

Mitochondria.—The mitochondria are usually spherical to ovoid, or, less commonly, elongate. They have protozoan-type tubular cristae which are occasionally seen to be continuous with the inner mitochondrial membrane.

Microbodies.—Microbodies are constant cytoplasmic organelles present in all specimens examined (Pl. 3, fig. A, Pl. 6, fig. A, Pl. 7, figs. C, D and E, Pl. 8, fig. B). We refer to these organelles as microbodies, because of their morphological resemblance to hepatic microbodies (Shnitka, 1966). The *Gromia* microbody in its typical form is spherical to ovoid, 0·5–1·0 μm in diameter, with a dense granular matrix and a single limiting membrane (Pl. 3, fig. A, Pl. 7, fig. C). In the matrix there are up to 4 tubular structures, or " nucleoids ", 30–35 nm. in diameter, with a wall thickness of 8 nm. The tubules when more than one is present lie approximately parallel to each other, the distance between each being variable. There is variation from this general pattern, especially in shape—some microbodies having an irregular outline (Pl. 8, fig. B). The density of the matrix is slightly variable (Pl. 7, fig. E), and occasionally the nucleoids vary from the simple tubular pattern (Pl. 7, fig. D). The variant nucleoids are ill-defined, slightly-denser, regions of the matrix. Microbodies are found throughout the cytoplasm and are not spatially related to any particular part of the cytoplasm. The single limiting membrane is rarely resolved as a tripartite structure, but when resolved it varies from 6–6·5 nm. in thickness.

Similar microbodies are present in Foraminifera (Hedley *et al.*, 1967, and Hedley & Wakefield as a personal communication in, Hruban & Rechcigl, 1969, and unpublished observations). All Foraminifera so far examined (*Allogromia laticollaris, Allogromia sp. Boderia turneri, Cibicides lobatulus, Elphidium macellum, Iridia lucida, Rosalina leei* and *Shepheardella taeniformis*) are marine, intertidal or shallow-water forms, with the same habitat as *Gromia*, and all possess microbodies. Microbodies in Foraminifera differ from species to species, but within each species are morphologically constant. In *Boderia turneri* (Lagynidae) the microbodies have a simple nucleoid

consisting of 1–4 tubules, and are almost identical in morphology to *Gromia* microbodies. The only difference being that those of *Gromia* have a variable shape.

Recent biochemical studies on microbodies from rat liver and kidney have established the following enzymes as constituents of these organelles: catalase, D amino acid oxidase, L α hydroxy acid oxidase, uricase (liver only) and L amino acid oxidase (kidney only) (Baudhuin *et al.*, 1965). It has been suggested that these microbodies are important sites of hydrogen peroxide metabolism and they have been renamed peroxisomes (De Duve & Baudhuin, 1966). Peroxisomes are characterized by: (a) the presence of oxidases and catalase, these enzymes being linked in catalyzing the reduction of oxygen to water; and (b) morphologically as small discrete particles limited by a single membrane. Although *Gromia* and foraminiferan microbodies have a close morphological similarity to hepatic peroxisomes, the lack of any knowledge of their biochemistry precludes their inclusion as peroxisomes. In this context it is interesting to note that the above authors (Baudhuin *et al.*, 1965) recovered a particulate fraction from the protozoan *Tetrahymena pyriformis* which contained peroxisome enzymes.

Golgi apparatus.—Relatively large, well developed Golgi organelles, with a constant morphology are common throughout the cytoplasm (Pl. 8, fig. A). A branch of the endoplasmic reticulum is always present along the convex or proximal face (= forming face of Waley, 1966). The region between the endoplasmic reticulum and the first cisternae is occupied by many small vesicles, 30–100 nm. in diameter. The cisternae near the concave or distal face (= maturing face of Waley, 1966) are distended and contain small vesicles. The concavity formed by the curved cisternae is often occupied by a large vacuole containing vesicles and a poorly-defined fibrillar material (Pl. 8, fig. A). Similar vacuoles are present elsewhere in the cytoplasm, where they are not associated with a Golgi apparatus (Pl. 8, fig. D). Some of these vacuoles are seen to be adjacent to (Pl. 8, fig. C) and in continuity with the plasma-membrane, their contents being in contact with the fibrillar material of the shell (Pl. 9, fig. A). It is tempting to suggest that these vacuoles, in three different positions, at the plasma-membrane, free in the cytoplasm, and closely associated with the Golgi apparatus, represent stages in a continuous process. The two end points in this process are the Golgi apparatus and the plasma-membrane. The vacuoles are formed either at the plasma-membrane, move into the cytoplasm and become associated with the Golgi apparatus, or, are formed at the Golgi apparatus move through the cytoplasm and fuse with the plasma-membrane. The direction of movement is not known. A brief consideration of other cells, where some of the functions of the Golgi apparatus are reasonably well established, may be useful here. In Brunner's gland of the mouse (Friend, 1965), goblet-cells of the rat colon (Neutra & Leblond, 1966), and wheat seedlings (Northcote & Pickett-Heaps, 1966) one of the known functions of the Golgi apparatus is the synthesis of polysaccharide, which is subsequently exported from the cell. A function of the Golgi apparatus in exocrine pancreatic cells of the guinea-pig is to concentrate enzyme, and enzyme precursors, into zymogen granules which are also exported (Caro & Palade, 1964, and Jameson & Palade, 1967). In rat transitional epithelium the Golgi apparatus produces a specialized thickened plasma-membrane (Hicks, 1966). The particular function of

the Golgi apparatus in a particular cell will be dependent on the function of that cell, but as the above examples indicate these fit into a generalized pattern of secretion, carbohydrate synthesis and subsequent export from the cell. This evidence from other cells suggests a direction of movement for the vacuoles in *Gromia* i.e. from Golgi apparatus to plasma-membrane. The opposite direction has been suggested to occur in amoeba (Daniels, 1964), but subsequent studies, using alcian blue as label, indicate that the endocytic vacuoles become food-vacuoles and do not contribute to the Golgi apparatus (Hayward, 1963, Chapman-Andreson & Nilsson, 1967). Assuming a direction of movement of vacuoles from Golgi apparatus to plasma-membrane the details of the process in *Gromia* are probably as follows: Protein together with polysaccharide precursor is budded off from the endoplasmic reticulum in vesicles around the proximal face of the Golgi apparatus. These vesicles coalesce and form the proximal cisternae. The polysaccharide precursors are polymerized into polysaccharide which with protein is organized into wall building units. As this takes place the cisternae move across the Golgi stack as other cisternae leave the distal face and more are formed at the proximal face. Near the distal face, the cisternae dilate forming vacuoles. These are released into the cytoplasm, migrate to the plasma-membrane, fuse with the plasma-membrane, and discharge their contents.

A feature of this process worthy of discussion is the variation in membrane morphology of the various components. The membranes of the vacuoles and contained vesicles are 8 nm. in width, tripartite and not heavily stained—β cytomembranes (Sjostrand, 1967). The membranes of the endoplasmic reticulum and Golgi apparatus are 6·5 nm. in width, electron dense and rarely resolved as tripartite—α and γ cytomembranes (Sjostrand, 1967). These membranes are structurally distinct (Sjostrand, 1963a, b, c), the α and γ cytomembranes having a globular substructure, and the β cytomembranes having a laminar substructure, and it has been suggested that transformation from one type to another does not occur (Ledbetter, 1962). A similar membrane change occurs in those instances cited above. For example, zymogen granules, transitional epithelium membrane and the vacuoles in the cells of Brunner's gland all have β cytomembranes and all originate from the Golgi apparatus—γ cytomembranes. Membrane variation within the Golgi apparatus has been described by Grove *et al.* (1968)—the membranes of the proximal cisternae are lightly stained and not resolved as tripartite, those of the distal face are densely stained and clearly tripartite, and the intercalary cisternae vary between these two extremes. From these examples it appears that transformation from γ cytomembranes to β cytomembranes is possible.

Secretion granules.—In the peripheral cytoplasm and in the cytoplasm around the oral region there are bodies not seen elsewhere in the cytoplasm, and which we shall refer to as " secretion granules ". They are spindle shaped 2–3 μm in length, 1–1·5 μm in width, limited by a single membrane and possess a dense granular matrix differentiated into two regions (Pl. 9, fig. B and D, Pl. 10, fig. A). One of these regions is in the form of a rod, 0·2 μm in width, and extends the length of the granule. (Pl. 9, fig. B, Pl. 10, fig. A). The second region is composed of electron-dense granules, 8–10 nm. in diameter, tightly packed and arranged in concentric layers (Pl. 10, fig. A). The third region is an electron-dense reticular body composed of

small granules 10–15 nm. in diameter (Pl. 9, fig. D). Although nothing is known about these granules beyond their morphology, two observations indicate their possible function. Firstly, the contents of the granules often appear to have contracted away from the limiting membrane, giving the granule the appearance of a vacuole-contained body. Secondly, many of the granules have lost most of their internal contents, apart from the granular regions, which appear to be breaking down to produce a fibrillar material (Pl. 10, fig. C and D). We consider it likely that this is the fibrillar mucous material which eventually lines the pseudopodia.

Xanthosomes and stercomata.—Xanthosomes and stercomata are hard brittle granules composed of concreted waste—material and are present throughout the cytoplasm (Pl. 6, fig. A). They have been discussed in a previous publication (Hedley & Bertaud, 1962) to which nothing new can be added at present.

Pseudopodia.—A lucid description of the pseudopodia and their activity in living *Gromia* is given by Jepps (1926): " The pseudopodia are perfectly hyaline during life, no granules being visible in them even under an oil immersion lens. They emerge very slowly, as a little bunch of stiff slender spikes, from an area of the cytoplasm lying just inside the mouth of the shell and free from ingesta. They extend in all directions, gently waving about as if in search of something of which to take hold. When a pseudopodium comes up against a solid object it sticks to it and flows out over it. As the pseudopodia increase in size they branch, and become irregularly varicose and ridged so that they look somewhat like the gnarled roots of a tree. From swollen places or knots there arise fresh pseudopodia extending at wide angles and often anastomosing with others in the vicinity. Some may creep back over the surface of the shell: otherwise there is no extramural protoplasmic layer." It must be emphasized that movement of the pseudopodia is extremely slow and especially so when compared with that encountered in Foraminifera. Movement is of two types, first, of a pseudopodium as a whole, and secondly, of protoplasm within a pseudopodium. Once an animal has begun to extrude pseudopodia at least 20 minutes is required for their full extension (Pl. 1). When fully extended, movement of a pseudopodium as a whole is rare. Movement of protoplasm along an extended pseudopodium has not been seen, although specimens have been examined using bright field, phase contrast, interference and polarizing microscopy.

For fine structure studies pseudopodia were fixed in a variety of fixatives (see Material and Methods) in each of the following conditions: (i) whilst being extruded; (ii) when fully extended; and (iii) when contracting, due to release of the animal from the substrate. In each case the fine structure of the pseudopodia was the same, consisting of ground-plasm, limited by a membrane, and surrounded by mucus (Pl. 10, fig. E). At higher magnification (Pl. 11, fig. A), the membrane is seen to be tripartite, 9·5 nm. in width, and the ground-plasm amorphous. The only formed elements within the pseudopodia are rare vesicles and dense bodies of unknown nature and function (Pl. 11, fig. C). The mucous layer, approximately 1 μm thick, is fibrillar with the fibrils parallel to the length of the pseudopodium (Pl. 11, fig. C).

There is no discernable difference in fine structure along the length of the pseudopodia from the oral capsule to the distal extremities. On the cytoplasmic side of the oral capsule however, the ground-plasm of the pseudopodia is more condensed. The

pseudopodia arise from the cytoplasm in this region at approximately 100–200 μm from the oral opening. The junction of the pseudopodia with the cytoplasm is very sharp (Pl. 11, fig. D, Pl. 12, fig. A). and there is no discernable structure associated with this abrupt change. The mucous lining to the pseudopodia is present in this region (Pl. 12, fig. A) and most likely originates from mucus-containing vesicles, which are concentrated in the oral cytoplasm (Pl. 12, fig. A). Where these originate is not known, although there are indications that the " secretion granules " may break down to liberate the mucus (Pl. 10, fig. C and D). Such granules are present in large numbers in the oral region.

It is useful to compare the pseudopodia of *Gromia* with recent accounts of pseudopodia in Foraminifera (Wolfarth-Botterman, 1961, Hedley *et al.*, 1967) and Testacea (Berrend, 1964, Wohlman & Allen, 1968). The enigmatic nature and taxonomic significance of the pseudopodia of *Gromia* have been discussed briefly (Hedley, 1964). The pseudopodia of Foraminifera are, at the optical microscope level, similar throughout the group. They form a complex anastomosing network of rapidly moving pseudopodia with each strand supporting two streams of oppositely directed granular protoplasm moving at about 8–15 μm per second (Allen, 1964). Electron microscopy of the pseudopodia has shown that mitochondria, vesicles (Wolfarth-Botterman, 1961, and Hedley *et al.*, 1967) and microtubules (Hedley *et al.*, 1967, and unpublished observations) are present. The microtubules may be few or may fill the cross section of a pseudopodium, and, unlike those in Heliozoa (Tilney *et al.*, 1966), have a random arrangement. This is very different from *Gromia* whose pseudopodia move extremely slowly, have no granules, have no perceptable movement within the pseudopodia, and whose fine structure is characterized by an absence of any formed elements.

Microtubules are labile cell constituents (Porter, 1966), and their absence in *Gromia* pseudopodia could be due to any of the following reasons: (i) they were lost on fixation; (ii) they were not present in that part of the pseudopodium examined, at the time of fixation, but may have been present at some earlier time when the functional state of that part of the pseudopodium was different; or (iii) they are not normal constituents of the pseudopodia. It is doubtful if the absence of microtubules is due to the first two reasons, as the fixation procedures used usually preserve microtubules, and the pseudopodia were fixed in three functional states—extending, fully extended and retracting. Many sections of each were examined and microtubules were not demonstrated. It would appear that microtubules are not normal constituents of pseudopodia in *Gromia*.

Microtubules are usually considered to have a cytoskeletal function, and are common constituents of fine cellular extensions (Porter, 1966). Their absence in *Gromia* pseudopodia indicate that the rigidity of these structures is due to other unknown factors. Cytoplasmic streaming has not been observed in *Gromia* pseudopodia, and filaments approximately 5 nm. in diameter, commonly found in streaming cytoplasm (Nachmias, 1968, Wolpert *et al.*, 1966), and often considered as providing the force for cytoplasmic streaming (Wolpert, 1962), have not been detected.

The Testacea are a more diverse group than the Foraminifera, there are two main types of pseudopodia within the group, lobopodia and filopodia. In *Difflugia*

(Testacealobosia, De Saedeleer, 1934) there are usually 1–4 lobopodia with small side-branches. Pseudopodial movement is rapid and granular streaming is present within the pseudopodia, the speed of movement ranges from 2 μm to at least 15 μm per second (Wohlman & Allen, 1968). The fine structure of the pseudopodia includes vesicles, electron-dense granules and extensive tracts of microfilaments (Wohlman & Allen, 1968). In *Cyphoderia* (Testaceafilosa) there are usually 5–6 filopodia with small side-branches, pseudopodial movement is rapid, and the speed of movement of protoplasm within the pseudopodia is about 4–5 μm per second (Berrend, 1964). The two types of testacean pseudopodia are quite different from *Gromia* pseudopodia.

GAMETES

The main observations of earlier biologists concerning gametogenesis and syngamy (Wright, 1861; Schaudinn, 1894, 1899; Zarnik, 1907; Awerinzew, 1910; Lwoff, 1925) have been reviewed by Jepps (1926). She was the first worker to describe nuclear division and the flagellated gametes. Further illustrations of gametes have been given by Le Calvez (1938) and Hedley (1962). Arnold (1966) reported the pairing of adults with the production of isogametes, and has proposed a tentative life-cycle.

Gametes have a single mucronate flagellum, 10 μm in length (Pl. 12, fig. B and C), with no appendages. This is only half the length of the flagellum described from optical microscopy (Jepps, 1926).

Although the fixation of gametes for fine structure studies was poor and the micrographs are mostly unfit for publication they have been useful in determining the internal organization of the gametes (Text-fig. 4). The body of a gamete is 2–3 μm in diameter, roughly spherical and limited by a plasma-membrane with no outer coating or wall. The single nucleus, 1·5 μm in diameter, contains dense chromatin with a few irregularly-shaped less-dense regions. There are usually two mitochondria, several fat droplets, and a small poorly-defined body which may be a microbody. There is a single flagellum and two basal bodies arranged at right angles. Each flagellum contains nine pairs of peripheral and one pair of central tubules. The presence of a single flagellum and two basal bodies is usually thought to be due to the loss of a flagellum during phylogeny from an organism originally having two flagellae (Manton, 1965). Such reduction has occurred sporadically in flagellate plant-cells (Manton, 1965). The arrangement of the fibres in the tips of mucronate flagellae is not known (Manton, 1965). In *Gromia* it appears that the outer ring of fibres stops at the junction of the tip, and only the central pair continue into the tip (Pl. 11, fig. B). This arrangement is similar to that of *Micromonas pusilla*, which has a long hair point (Manton, 1959).

There are two regular and obvious components of gamete cytoplasm whose nature and function are unknown, neither of which has been seen in adult animals. The first is a spherical to ovoid structure, 1 μm in diameter, limited by a single membrane, and containing two types of ill defined structures (Pl. 12, fig. D, Text-fig. 4-X). The second structure is membraneous and its morphology poorly understood (Pl. 12, fig. E, Text-fig. 4-Y).

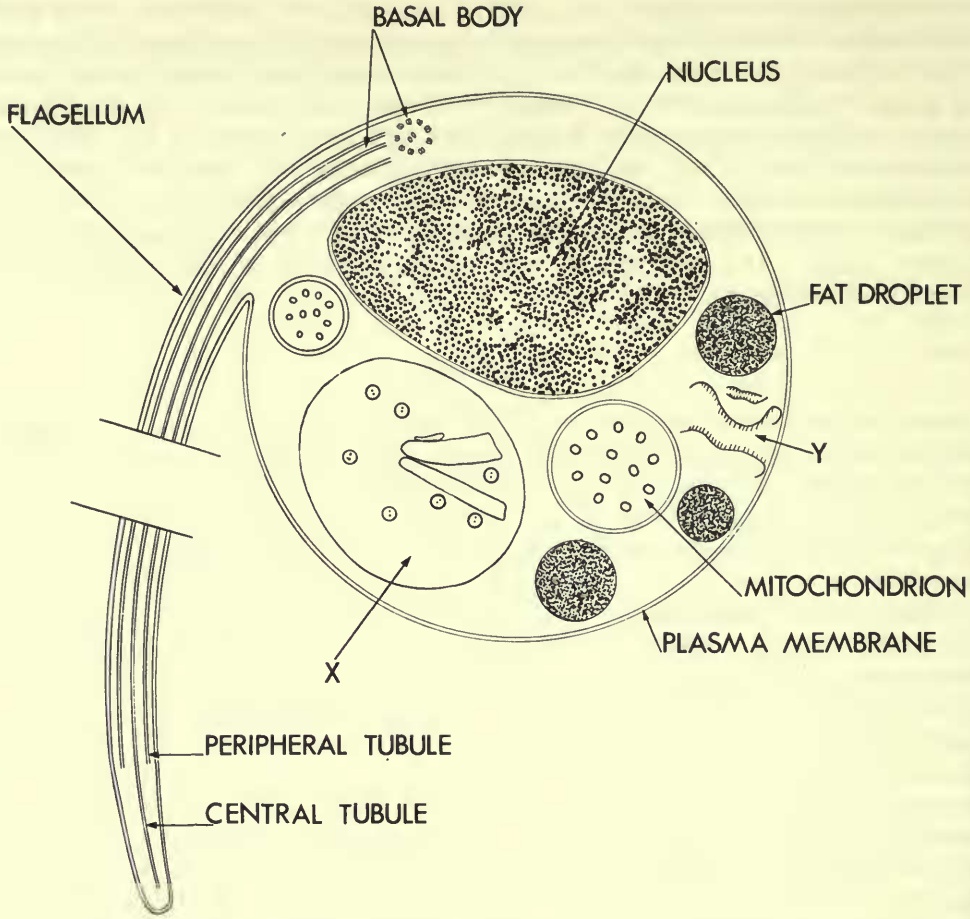

Fig. 4. Diagram of a gamete based on the examination of several micrographs. X, body of unknown function containing block-shaped and oval structures; Y, membraneous structure of unknown function. Both X and Y constituents are noted in the text

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RONALD HENDERSON HEDLEY, Ph.D.
Department of Zoology
BRITISH MUSEUM (NATURAL HISTORY)
CROMWELL ROAD
LONDON, S.W.7.

JAMES ST. JOHN WAKEFIELD, B.Sc.
Department of Zoology
BRITISH MUSEUM (NATURAL HISTORY)
CROMWELL ROAD
LONDON, S.W.7.

PLATE 1

An adult *Gromia oviformis* Dujardin, 1839, with pseudopodia extended. × 30.

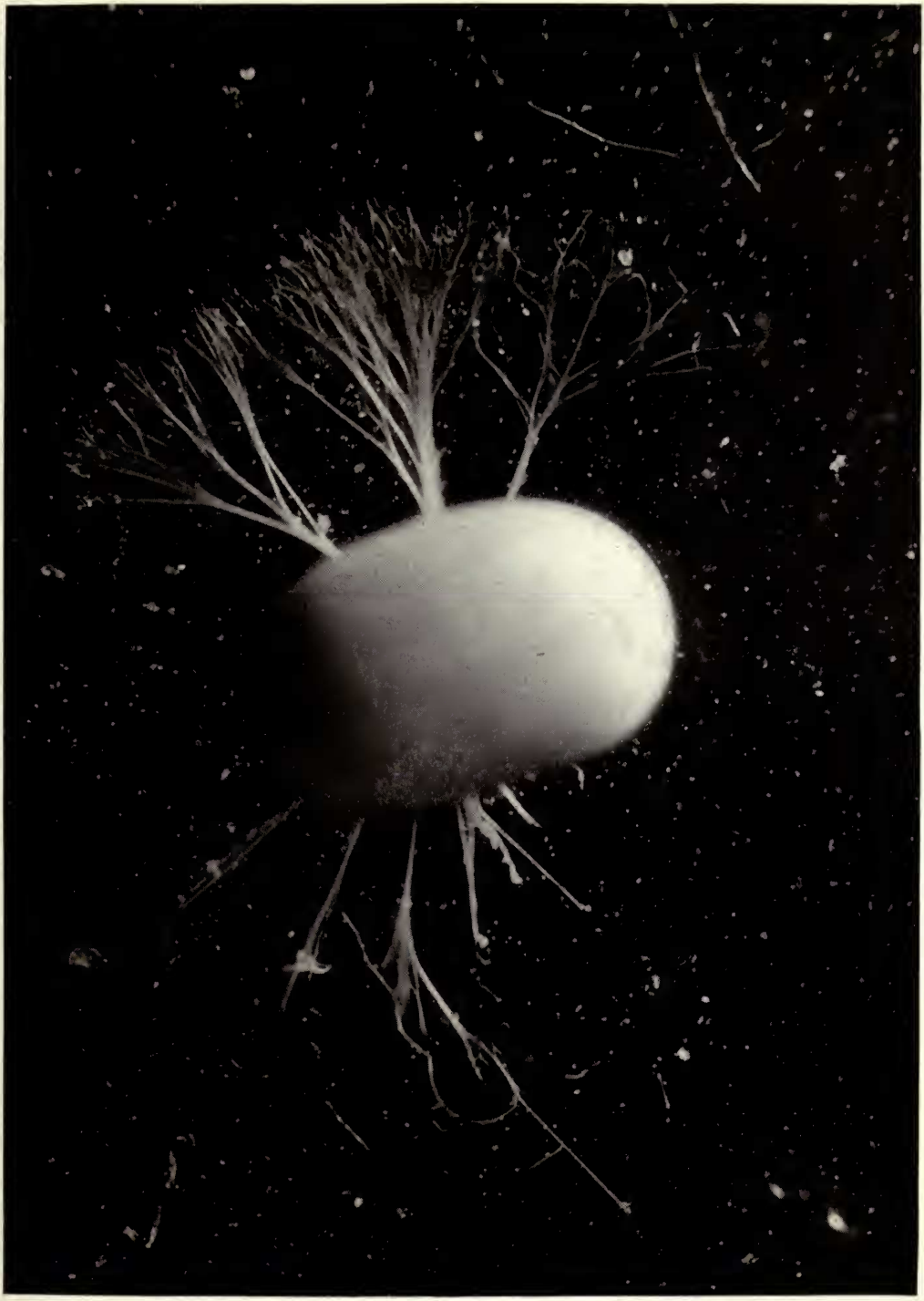


PLATE 2

FIG. A. Cross section through the shell and peripheral cytoplasm: EDL, electron-dense layer; OW, outer wall; HM, honeycombed membranes; F, fibrillar material; PM, plasma-membrane; C, cytoplasm. × 46,000.

FIG. B. Section of an outer wall canal illustrating the concentric arrangement of fibres. × 93,000.

FIG. C. Section through a wall canal: EDL, electron-dense layer; the arrow indicates concentration of electron dense spots referred to in the text. × 36,500.

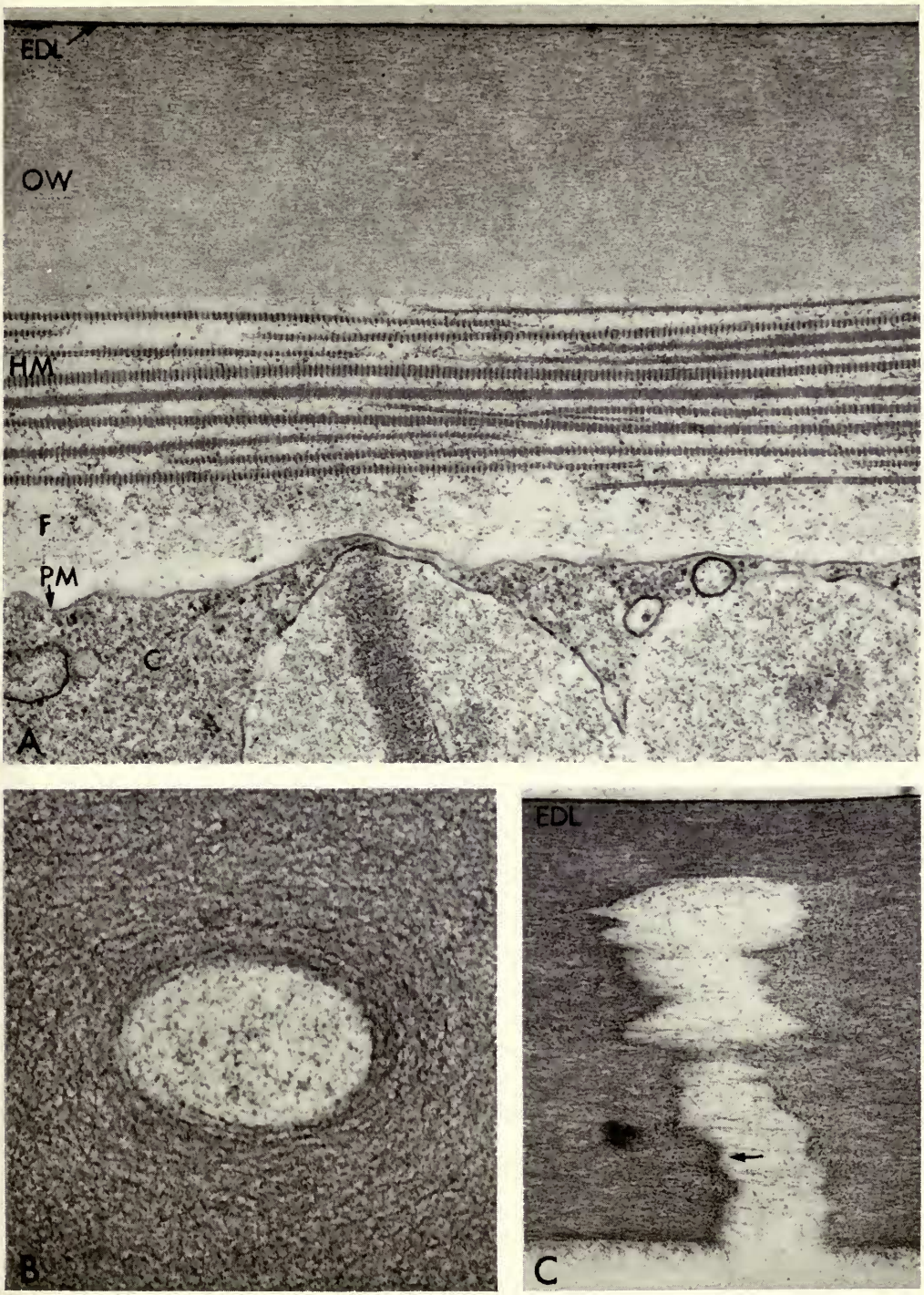

EDL
OW
HM
F
PM
C
A
B
EDL
C

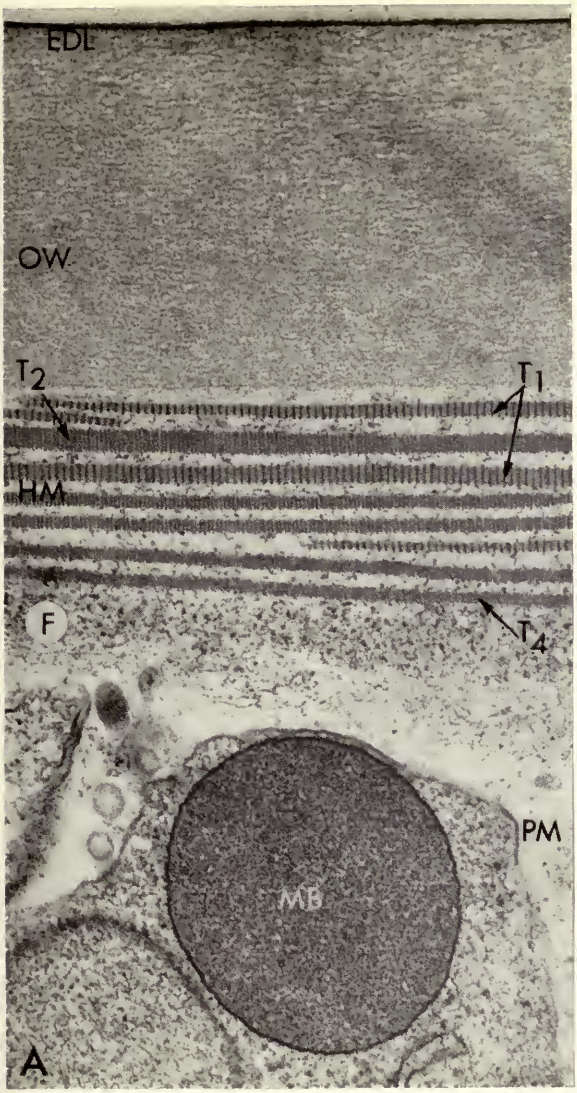
PLATE 3

FIG. A. Cross section through the shell and peripheral cytoplasm: EDL, electron-dense layer; OW, outer wall; HM, honeycombed membranes–T1 = type 1, T2 = type 2, T4 = type 4; F, fibrillar material; PM, plasma-membrane; MB, microbody. × 57,000.

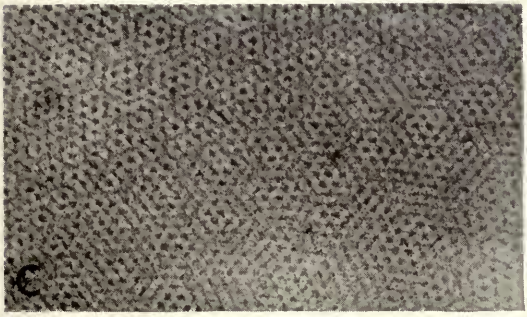
FIG. B. Cross section through the outer wall: EDL, electron-dense layer; P, pore. × 86,000.

FIG. C. A tangential section in which two honeycombed membranes have produced a Moiré effect. × 90,500.

FIG. D. Section through a canal pore (P). × 90,000.


EDL
OW
T2
T1
HM
F
T4
PM
MB
A


EDL
P
B


C

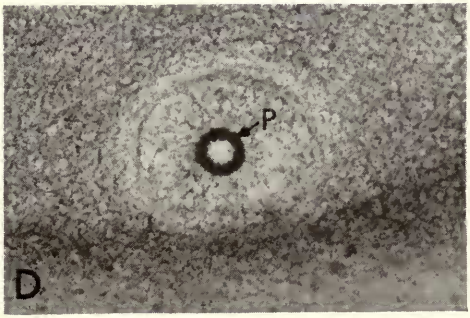

P
D

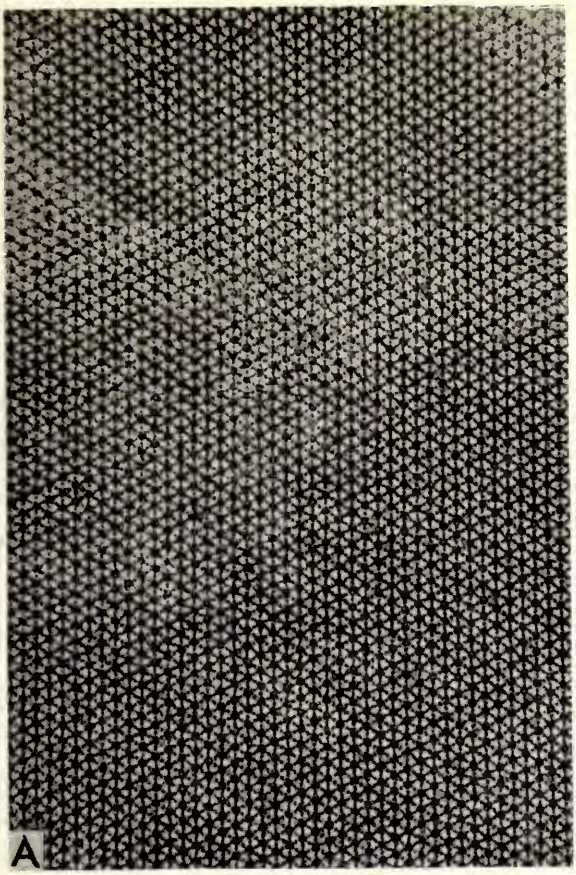
PLATE 4

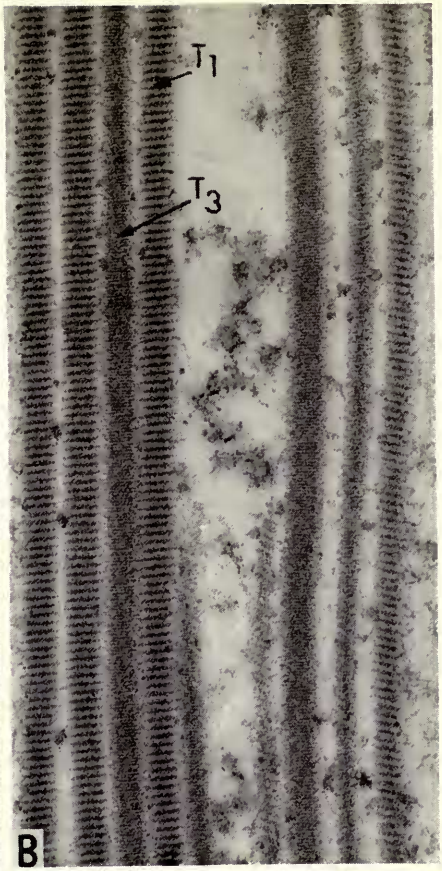
FIG. A. Tangential section of a honeycombed membrane. × 121,000.

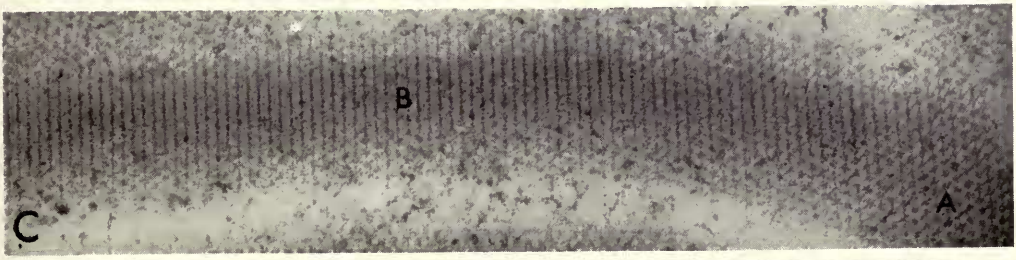
FIG. B. Cross section of honeycombed membranes: T1 = type 1, T3 = type 3. × 47,000.

FIG. C. Oblique section of a honeycombed membrane. At " A " the membrane is sectioned tangentially, at " B " it is sectioned nearly vertically. × 81,500.

FIG. D. Section through the microtubules of the oral capsule: M, microtubules; L, fibrils; LL, fibrillar layer. × 90,500.


A


T1
T3
B


B
A
C

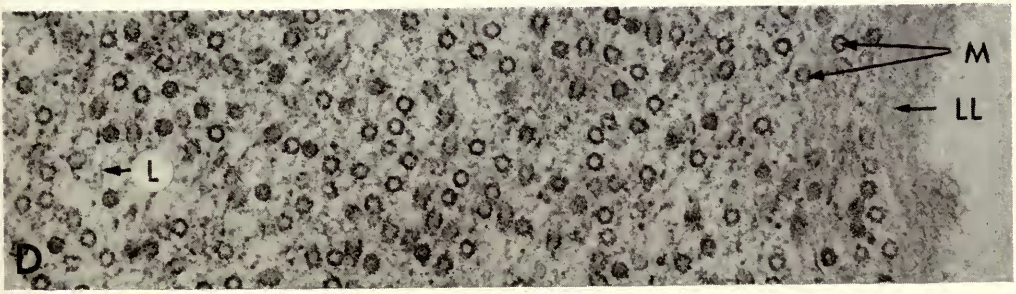

M
LL
L
D

PLATE 5

Cross section through the oral capsule (as in text-fig. 3): EDL, electron-dense layer; OW, outer wall; OW 1, outer extension of the outer wall; OW 2, inner extension of the outer wall; OW 3, thickening of the outer wall material at the junction; HM, honeycombed membranes; F, fibrillar material; PM, plasma-membrane; C, cytoplasm; M, microtubules; L, fibrils of the oral capsule. × 12,500.


EDL
EDL
OW1
L
M
OW3
OW2
F
PM
C
OW
HM

PLATE 6

FIG. A. General view of the cytoplasm: N, nucleus; NP, nuclear pore; MB, microbody; MT, mitochondria; S, stercomata; X, xanthosomes. × 13,000.

FIG. B. Section of the oral capsule stained with silver methenamine: EDL, electron-dense layer; OW, outer wall; M, microtubular mass—positive staining for polysaccharide. × 20,000.

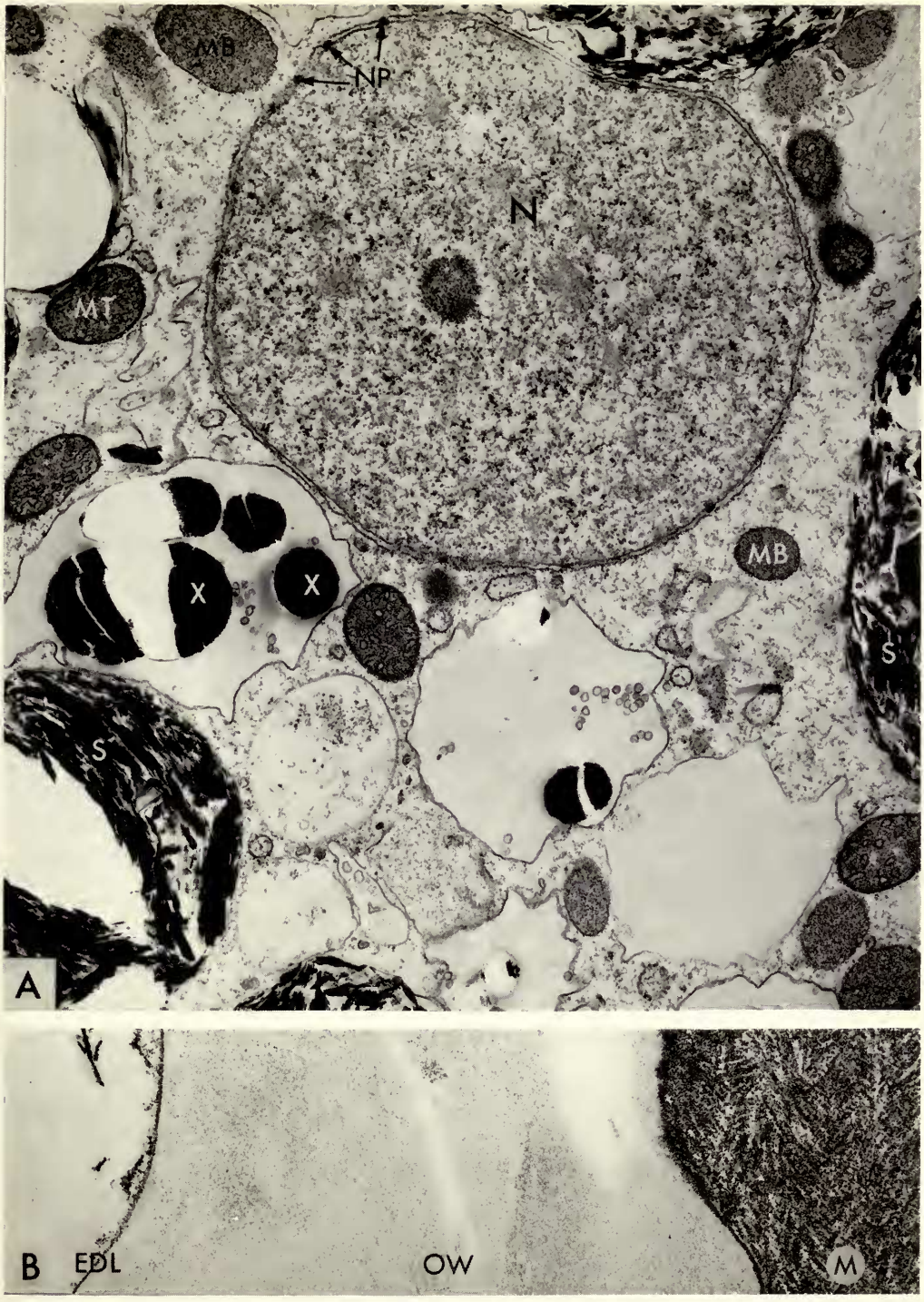

MB
NP
N
MT
X
X
MB
S
S
A
B
EDL
OW
M

PLATE 7

FIG. A. Part of a nucleus containing a nucleolus which is composed of two regions, G 1 finely granular and G 2, coarsely granular. × 8,500.

FIG. B. A tangential section of a nuclear membrane containing several nuclear pores, NP; P, polysomes. × 49,500.

FIG. C. A microbody with a single limiting membrane, dense granular matrix and a single tubule T. × 75,500.

FIG. D. A microbody containing two tubules T, and a variant nucleoid H. × 57,000.

FIG. E. Three adjacent microbodies showing variation in matrix density. × 36,000.

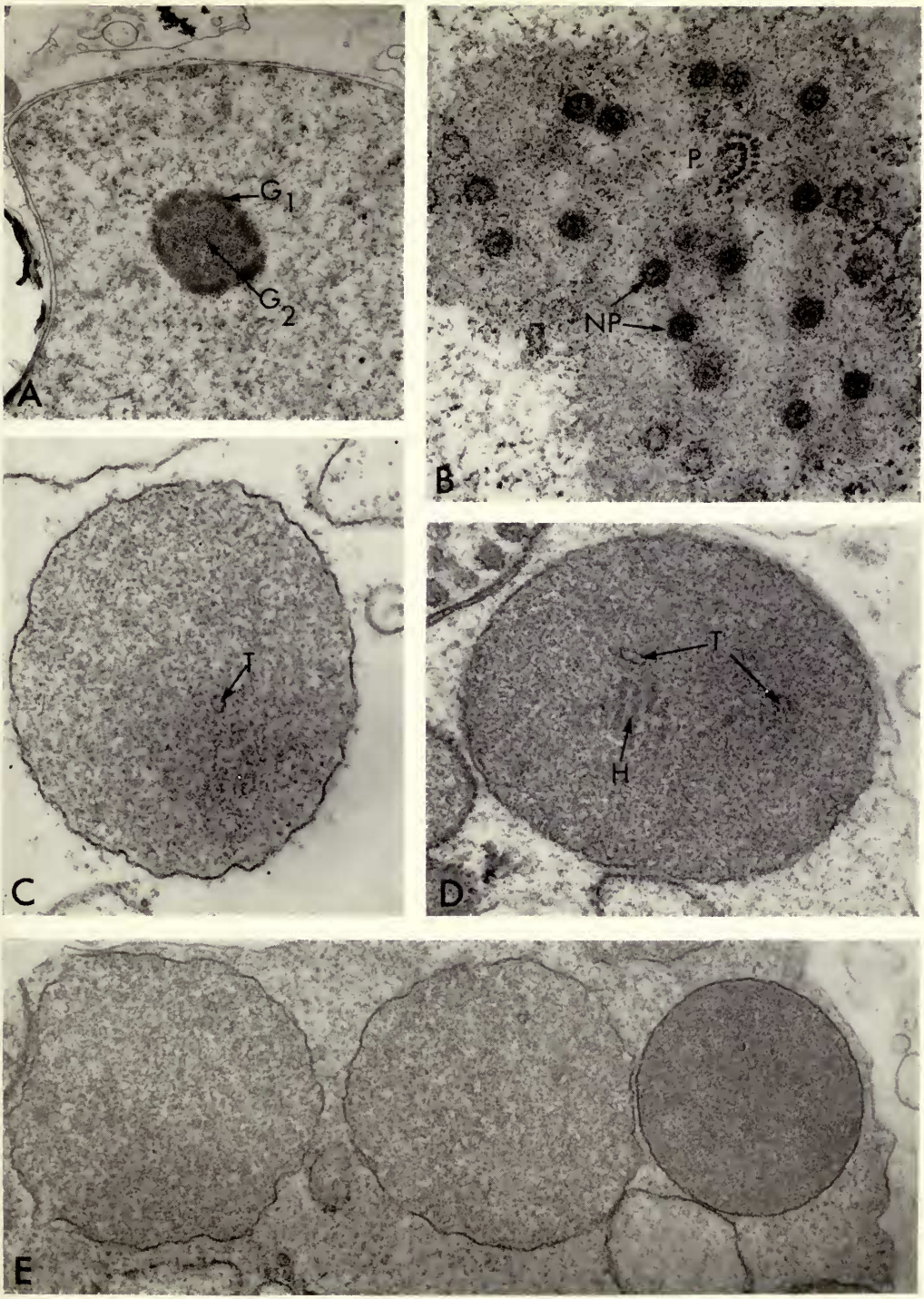

A
G1
G2
B
P
NP
C
T
D
T
H
E

PLATE 8

FIG. A. Golgi apparatus: ER, endoplasmic reticulum; V, vesicle; VO, vacuole containing fibrillar material and vesicles. × 55,000.

FIG. B. Microbody with irregular outline: T, tubules. × 60,000.

FIG. C. VO, vacuole containing fibrillar material and vesicles near the plasma-membrane, PM; HM, honeycombed membranes. × 60,000.

FIG. D. Vacuole containing fibrillar material and vesicles, free in the cytoplasm. × 59,000.

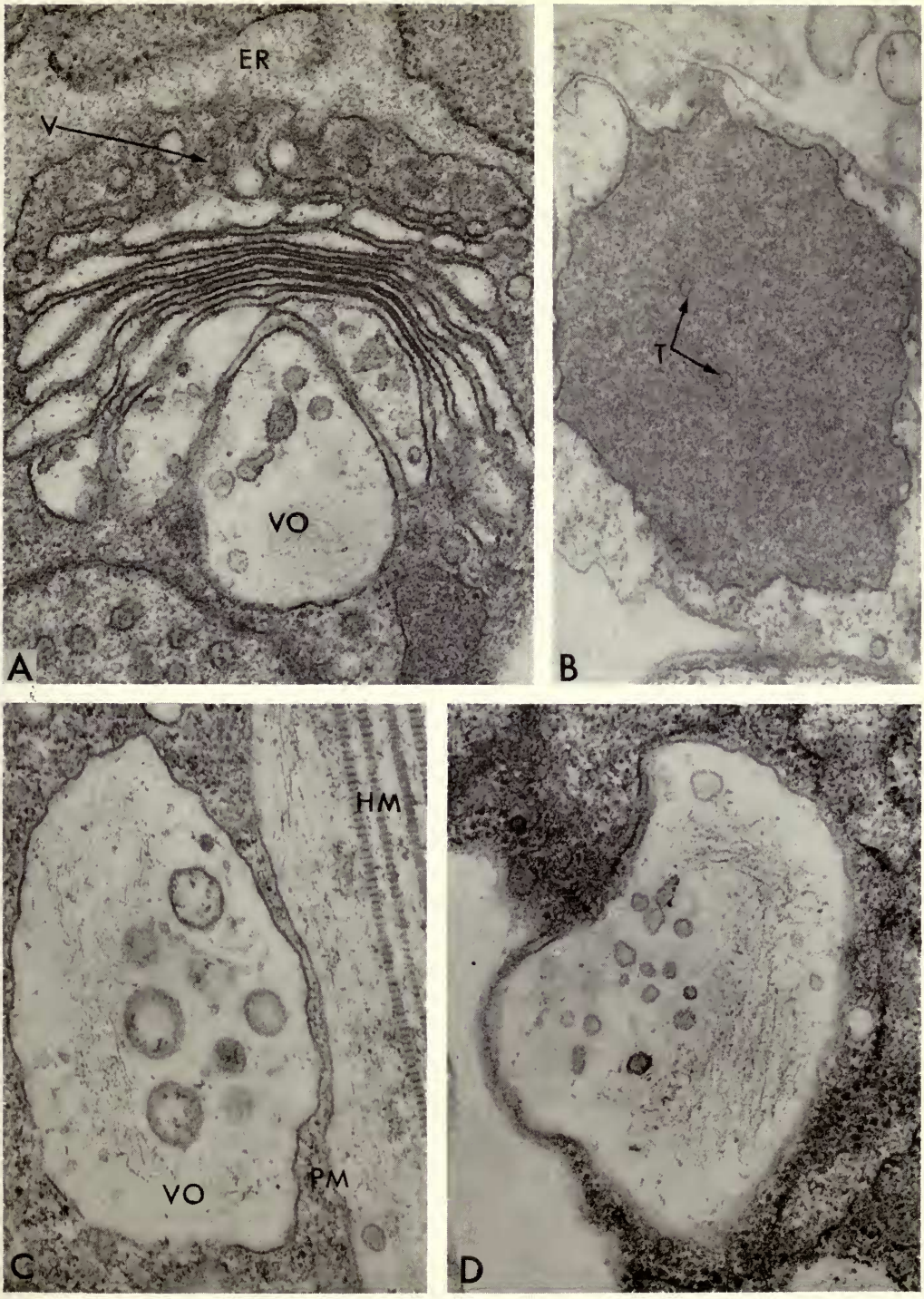

ER
V
VO
A
T
B
HM
VO
PM
C
D

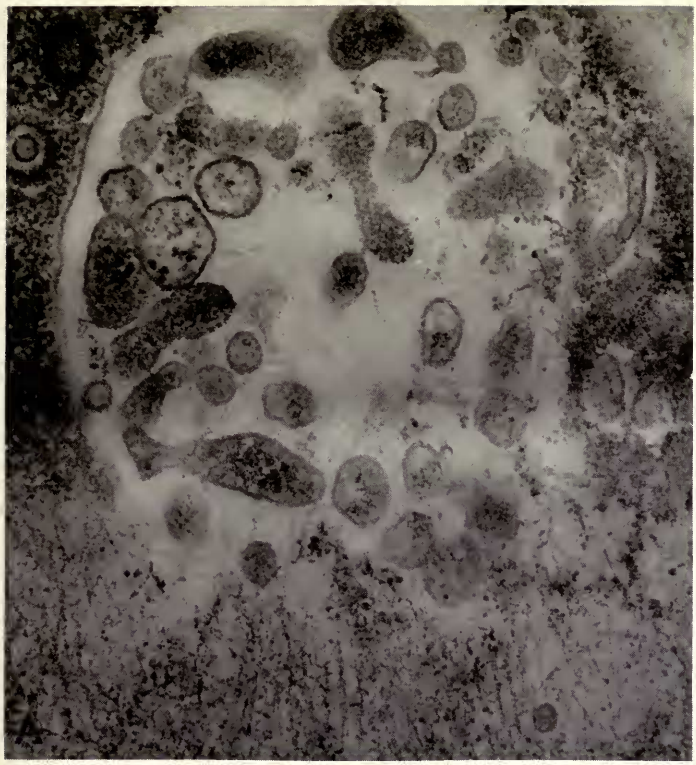
PLATE 9

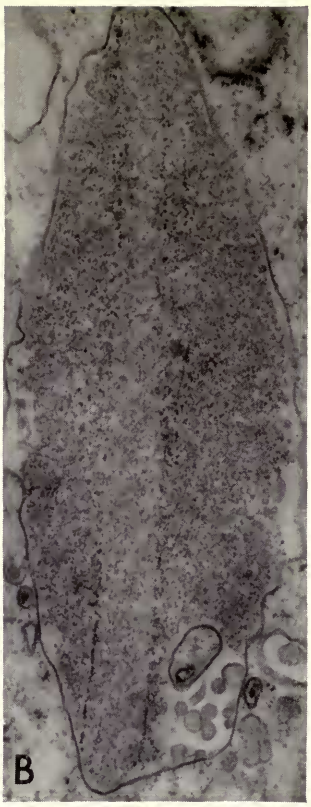
FIG. A. Vacuole in continuity with the plasma-membrane, discharging its content of fibrillar material and vesicles: F, fibrillar material of the wall. × 60,500.

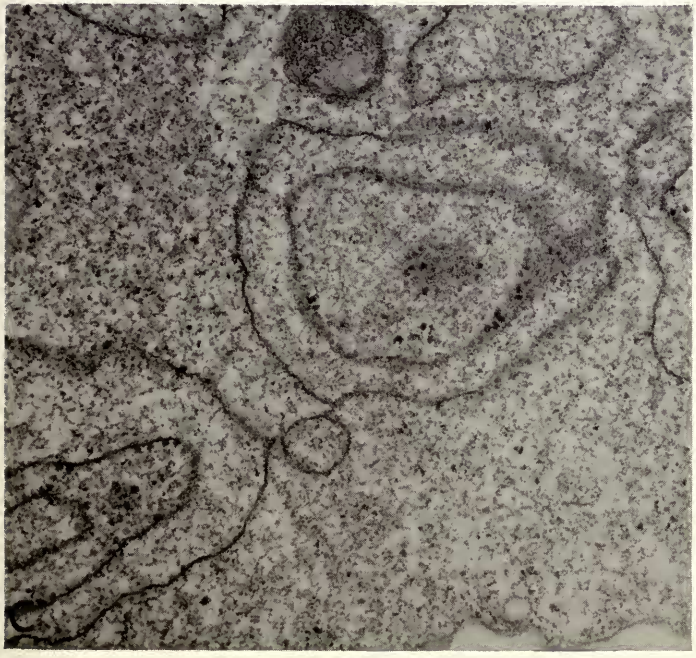
FIG. B. Secretion granule. × 35,000.

FIG. C. Section showing the distended cisternae of the endoplasmic reticulum. Note that the contents of the cisternae appear identical to the ground plasm. × 53,000.

FIG. D. Secretion granule with a differentiated region. × 26,500.




B



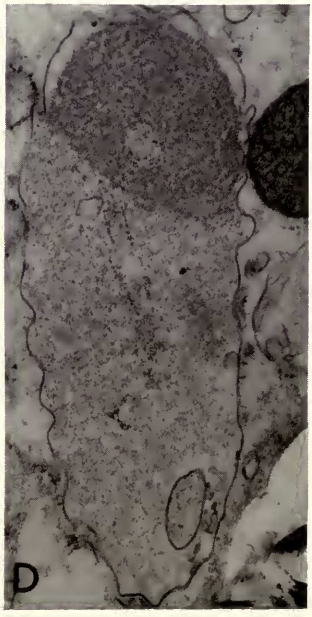

D

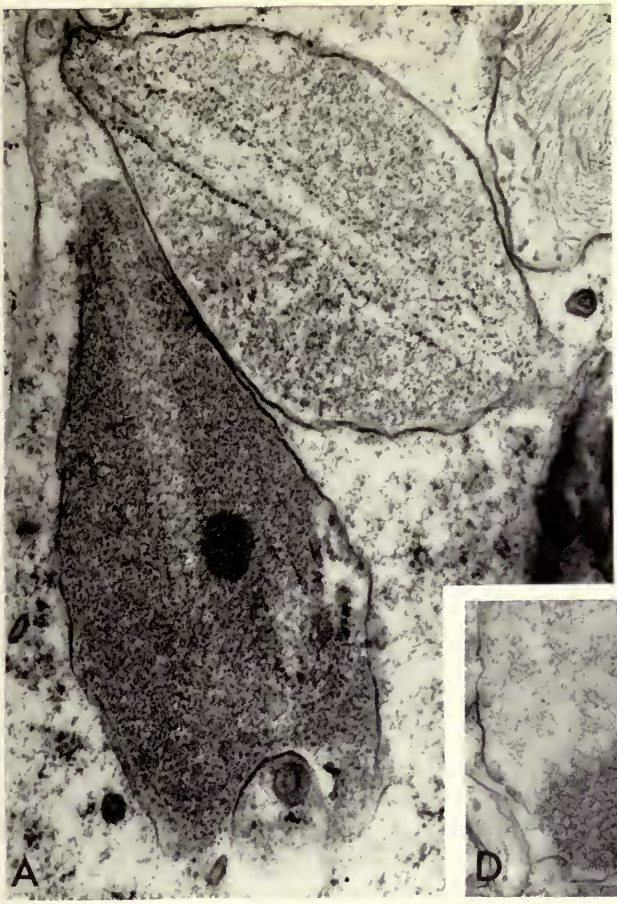
PLATE 10

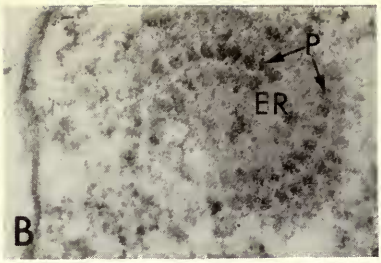
FIG. A. Two secretion granules, one with a central differentiated region. × 46,500.

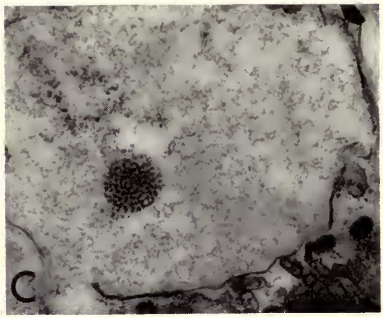
FIG. B. Tangential section of part of the endoplasmic reticulum, ER; with polysomes, P. × 96,500.

FIGS. C, D. Secretion granules breaking down to form fibrillar material. × 34,000.

FIG. E. Part of a pseudopodium: M, surrounding mucus; PM, plasma-membrane; G, ground-plasm. × 12,500.


A


P
ER
B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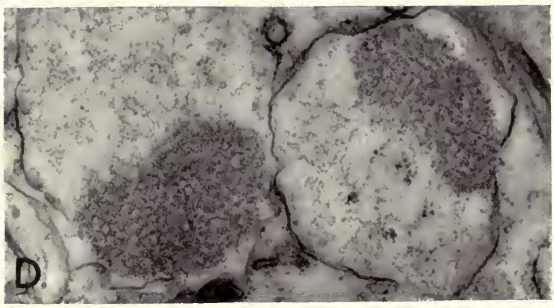

D

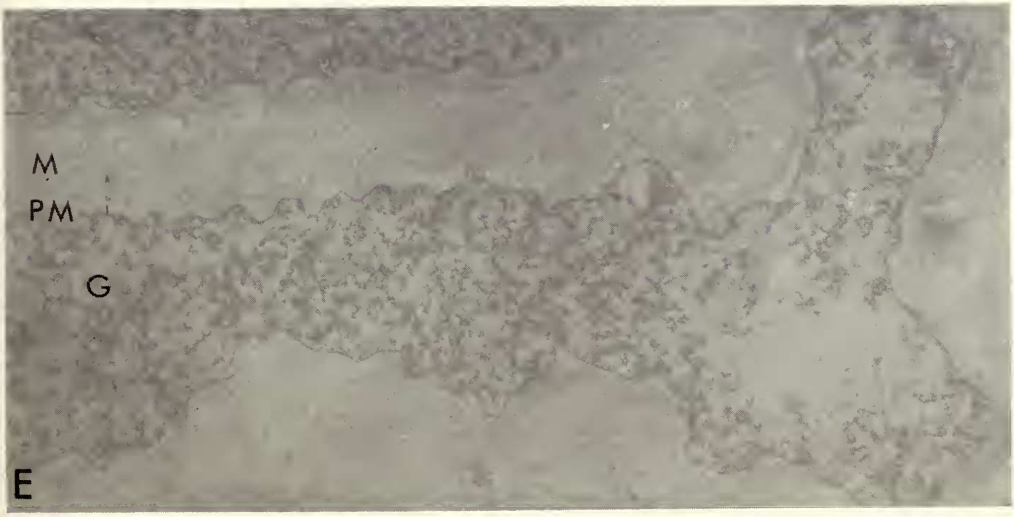

M
PM
G
E

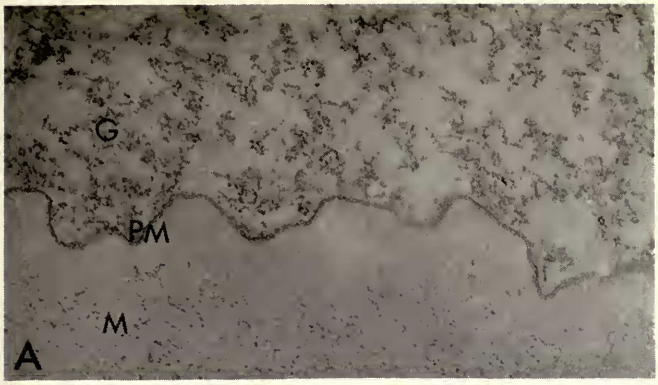
PLATE 11

FIG. A. Part of a pseudopodium: M, mucous layer; PM, plasma-membrane; G, ground-plasm. × 55,000.

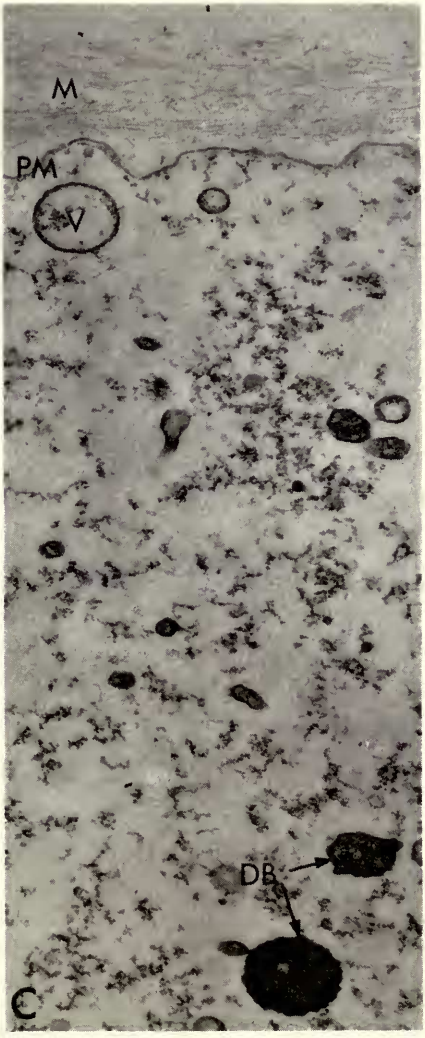
FIG. B. Section through the tip of a gamete flagellum: PM, plasma-membrane; PT, peripheral tubules; CT, central tubules. × 75,000.

FIG. C. Part of a pseudopodium: M, mucous layer; PM, plasma-membrane; V, vesicles; DB, dense body. × 25,000.

FIG. D. Junction of a pseudopodium and cytoplasm: PG, pseudopodial ground-plasm; C, cytoplasm; V, vesicles containing mucus. × 12,000.


G
PM
M
A


CT
PM
PT
B


M
PM
V
DB
C

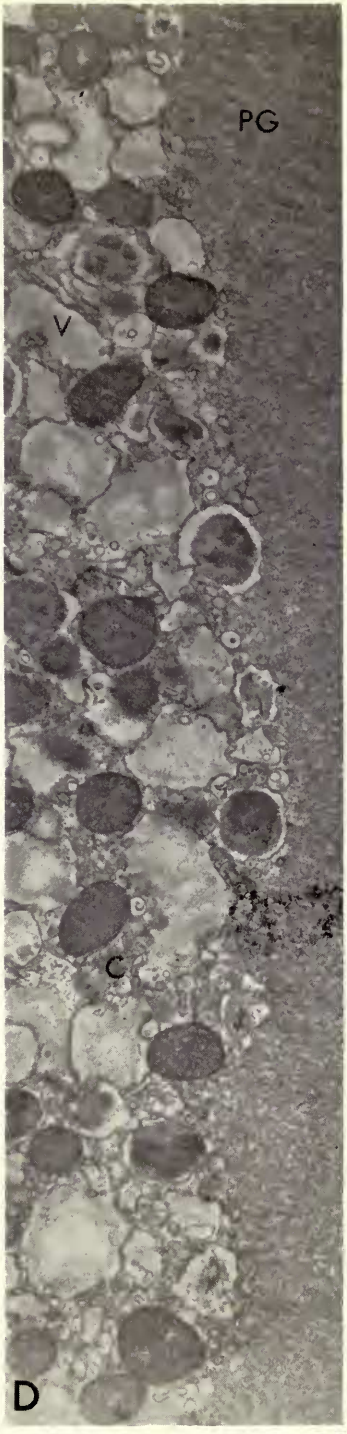

PG
V
C
D

PLATE 12

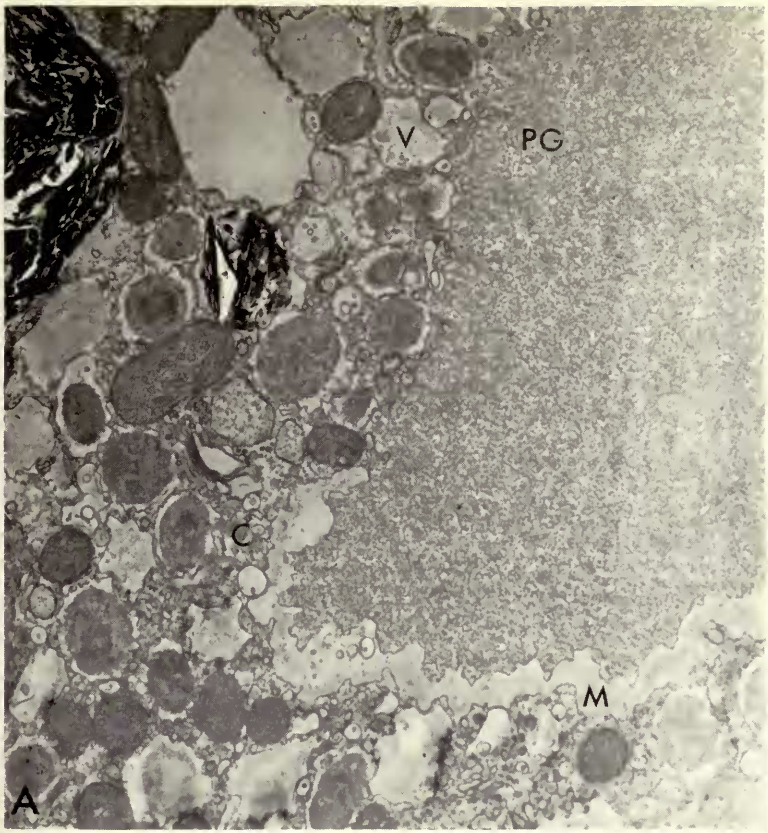
FIG. A. Junction of a pseudopodium and cytoplasm: PG, pseudopodial ground-plasm; C, cytoplasm; V, vesicles containing mucus; M, mucous layer separating the pseudopodium from cytoplasm. × 12,000.

FIG. B. Shadowed preparation of a gamete. × 7,000.

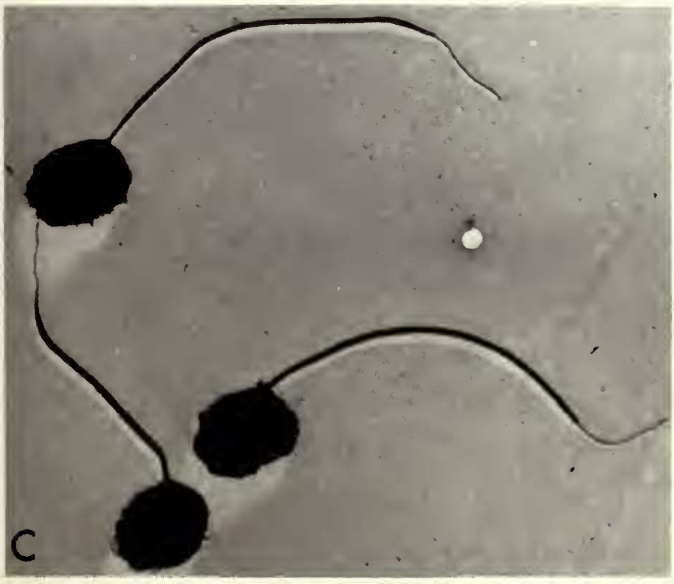
FIG. C. Shadowed preparation of gametes. × 6,000.

FIG. D. Enigmatic body present in gametes: BS, block-like structure; TS, tubular structure. × 28,000.

FIG. E. MS, membraneous structure present in gametes; PM, plasma-membrane. × 37,000.


V
PG
C
M
A


B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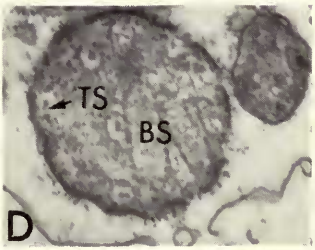
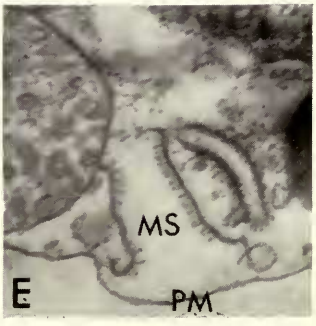

TS
BS
D


MS
E
PM